

Isolating and Diagnosing *Burkholderia Spp* from Soil and Soil Surrounding Onion Roots in Najaf Governorate

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Abstract

The present study included the isolation and identification of *Burkholderia* spp. samples were collected from places near soil and associated with plant onion roots ;rice farms;palm trees; *pinus*; *Frangula alnus* and *Medicago sativa*) in soil AL-Najaf province. All the samples were collected During a four- month from October 2020 to March 2021. *Burkholderia* isolates were isolated from (90) environmental sample from (soil).The isolates were identify according to morphology properties , cultural, conventional biochemical test then the diagnosis was confirmed by using Vitek 2 GN-card .*Burkholderia* spp isolates The isolates were obtained from soil and soil surrounding the onion root (Rhizospheres) for different plants group were S₄1(2.2)% and S⁵1(2.2)% .The researcher reached to that DNA sequencing is the best method for identification of bacteria and used of the best medium to growth *bacteria* it's Oxidation Fermentation Polymyxin Bacitracin Lactose Agar Base compared with used medium other.

PCR technique was used for the detection and amplification of (recA) gene in two *Burkholderia* spp. isolates and the results showed that all tested isolates harbor this gene, and analysis was investigated for confirming the identification of two selective *Burkholderia* spp. isolates under study. PCR product from the amplification of 16S rRNA gene were send for DNA Sanger Sequencing to Macrogen Company / Korea. The results of gene amplification and sequencing successfully confirm the identification and showed that two isolates belonged to *Burkholderia* spp. bacteria in similarity ratios (95)% with the *Burkholderia* spp. isolates in Gen Bank. After sequencing analysis, two isolates were deposited in GenBank was the two types species Mohammad(1) and Mohammad(2) were first recorded in Iraq, in Najaf province .

Burkholderia spp. isolate (S₄, S₅) was resistant towards Azithromycin , Augmentin and Amikacin (100%), while (S₅) sensitivity of isolates towards clavulanic acid, Meropenem and Cefazolin . While (S₄) sensitivity of isolates towards Tobramycin and Ciprofloxacin, Cefepime ; Ceftazidime.

Finally, the virulence factor of *Burkholderia* sp. all of the isolates were able to produce Gelatinase enzyme and biofilm isolates were tested. Positive results are demonstrated by partial liquefaction of the inoculated tube and inability to produce urease enzyme. The aim of study isolation and identification of *Burkholderia* Sp. from different environmental sources ,determination the bio patterns of isolates using Vitek 2 system and PCR., Molecular identification using diagnostic primers and using specialized primers to identify virulence factors for environmental and study of ten antibiotics and their effect on bacterial isolates and Determine the tolerance capacity of bacteria with study internal growth factors such as Temperature and PH.

In the molecular study, five genes related to virulence factors and resistant antibiotic were detected, namely PRND, OPCI, CciI, Bla-tem and PltC. Environmental samples possessed genes for the production of antifungal and antibacterial and the formation of purine proteins, and This explains the adaption Bacteria by habitat type in which they reside.

Keyword: *B. cepacia* ,PCR, Soil

1. Introduction

The genus *Burkholderia* comprises a wide variety of Gram-negative bacterial species, many of which are pathogenic to humans, plants and animals. The most pathogenic bacteria are *B. mallei*, *B. pseudomallei*, and the members of the *B. cepacia* complex (*Bcc*) (1). It comprises a subset of *Burkholderia* species associated with respiratory infections in people with cystic fibrosis especially pneumonia; bacteremia; UTIs and septic arthritis (2). and chronic granulomatous disease. *B. mallei* and *B. pseudomallei*, the cause of glanders and melioidosis, respectively (3). Most of the species in the genus are plant-associated bacteria even then. They are gram-negative bacilli and their shapes are between bacilli and short bacilli. Be poorly growing at 45 °C, The optimum temperature is (30-35)°C (4). Non-spore forming and produces non-fluorescent pigments. This bacterium is multi-nutrient; multi-metabolized, and it multiplies in distilled water; disinfectants; sterilizers, And herbicides are used as a source of nitrogen (5). In *Burkholderia* species, the number and size of replicons differ from strain to strain and the genome is very wide ranging from 6-9 Mb in size, this enormous genetic ability that demonstrates their flexibility in disease and natural biology (6), all species often divided this DNA into two or more chromosomal replicas, it can be concluded from this knowledge that the organisms have three chromosomes and one plasmid (7). Strains of bacterium could be isolated from plant roots rhizosphere surroundings environments, strains of *B. cepacia* differs in phenotypic features about those isolated from clinical samples (8). Between the differences were indole-acetic acid production, nitrogen fixation, phytopathogenic fungi, a wide temperature range all positive for rhizosphere strains (9). Numerous bacterium belong to the genus *Burkholderia* have PGPR (properties like context, cellulolytic, phosphate-solubilizing, regulating the elemental cycle and plant nutrition (10), Hence renewal the nutrient content in forest soils, these functional bacteria could have cross-functional abilities or beneficial traits that are necessary for plants and can improve their growth (11), so this functional bacterial strains could potentially be contained in bio-fertilizer formulations for crop growth on acid soil (12).

Soil microorganisms have an important influence on soil fertility and plant life, Interactions of *Burkholderia* species with soil microbes A possible survival strategy in acidic soils might be the close interaction with other soil microbes (13), as well as with higher organisms such as plants. there are numerous reports about interactions between *Burkholderia* and soil microbes, especially with fungi. Fungi are ubiquitous organisms but are, like *Burkholderia*, mainly associated with the soil environment community and it has been shown that their growth and activity decrease with increasing pH (14).

2. Materials and Methods

2.1. Specimens Collection

2.1.2 . Soil sample collection:- samples were collected from places near soil and associated with plant roots (Rhizospheres such as (rice farms, palm trees, or ornamental plants, pinus), using previously mentioned methods in source (15). were to determine locations that were associated with potentially higher presence of *Burkholderia* spp. such as animal pens, and agricultural activities carried out at the sites such as farming, bathing were also taken into our account during the survey. were dug within the vicinity of such locations to presence chances of *Burkholderia* spp. Depending on the size and the sampling site, were dug soil using to 30 cm depth. A hand trowel was then used to transfer soil into labelled zip-lock bags to The specimens were transported to the Advance bacteriology laboratory \ Microbiology department \ Faculty of Science \ University of kufa. The bags of soil were protected from direct sunlight as UV light had been shown to kill *Burkholderia* sp and the hand trowel were cleaned with water and sterilized with 70% ethanol, were taken one (gm) from the soil and added to 9ml of D.w. and after the work of Serial dilutions were prepared then was take 0.1ml of last dilutions and add

to culture media(MacConkey agar; Blood agar media and OFPBL Agar Base) and incubated at 37 °C for up to 48 hr (16).

2.1.3. Ready-Made Culture Media: The culture media used in this work with their purpose are listed in table (1).

Table(1): Culture's media used with their function in bacterial isolation.

NO.	Culture media	Manufacturing\ Country	Function
1.	Blood agar base	Hi-media\ India	Cultivation bacterial strains and to studies their capability of blood hemolysis
2.	Brain heart infusion broth	Hi-media\ India	This media was uses for detectation the capability of bacteria to produced biofilm formation.
3.	Cimmon citrate Agar	Hi-media\ India	Biochemical test
4.	Gelatin agar	Hi-media\ India	Used for gelatinase detection and gelatin hydrolysis
5.	Kligler iron agar	Hi-media\ India	Biochemical test
6.	MacConkey agar	Oxoid\ Paris ,France	Growth of G-ve bacteria and determining their capability to lactose fermentation
7.	MR-VP broth	Hi-media\ India	Biochemical test
8.	Muller-Hinton agar	Hi-media\ India	Antibiotic sensitivity test
9.	Nutrient broth and agar	Hi-media\ India	Cultivate and upkeep of non-fastidious bacteria
10.	Peptone water medium	Hi-media\ India	Detection of the capability of bacteria to produced Indole
11.	OFPBLE Agar Base	Prepare	Used for the primary identification of <i>Burkholderia cepacia</i>
12.	Cetrimide agar	Hi-media\ India	Ability Growth with cetrimide
13.	Urea agar base	Hi-media\ India	Biochemical test

2.1.4. Biochemical test:-The Biochemical tests were performed according to(17).

2.2.Optimization of parameters

2.2.1. pH optimum:-pH plays a very important role in growth and enzymes production which was required for growth of bacteria the biosynthesis, different pH values ranging from (5.0 ,6,0 and 9.0and 10) were adjusted in a series of 3 plastic screw cap tubes containing 10ml of nutrient broth medium from *Burkholderia* Spp. HCl was used to adjust pH <7, and NaOH was used to adjust pH>7. All the

tubes were incubated in a shaking incubator at 37°C for 24 hours and after then recorded the resulting (18).

2.2.2. Temperature optimum:-Temperature is an essential factor affecting of the grow the bacterium ,where study carried out using with temperature gradient ranging from (30,35, 42 and 46) °C tube then take of the test tubes and place it in the form with plastic screw for size ten ml contain ten ml of nutrient medium broth after the bacteria *Burkholderia* Sp.has planted in test tubes then all of tubes incubated were in a shaking incubator after then recorded the results (19).

2.3. Virulence factors

2.3.1. Biofilm formation on congo red agar:Each isolate's pure single colony was streaked on Congo red agar plates and inoculated at 37°C for 24-48 hours. Black colonies with a dry crystalline quality indicated positive results. The negative result was indicated by a darkening of the colonies in the absence of a dry crystalline colonial morphology (20).

2.3.2. Gelatin liquefaction:-Inoculate the gelatin deep with four to five drops of a 24-hour broth culture. Incubate at 35°C to 37°C in ambient air for up to 14 days., inoculate the gelatin deep from a 24-hour-old colony by stabbing four or five times, 0.5 inch into the medium.. Remove the gelatin tube daily from the incubator and place at 4°C to check for liquefaction.

2.4. Identification of *B.cepacia* by using VITEK 2 compact system and identification cards of Ge-ve bacteria.

The VITEK® 2 Compact system is dedicated to the identification of clinically significant bacteria. The system includes the VITEK® 2 Compact instrument, a computer, and printer. The software provided with the VITEK® 2 Compact system includes analysis and data-management programs. It is analyst automated diagnostic apparatuses confirmatory used for isolates using the card ID- GNB, has this card is designed for rapid diagnosis of the types of bacterial negative gram stain, and that the diagnosis of this card depends on the biochemical methods and develop new foundation materials, biochemical tests of different measurement sources of carbon consumption result and takes about 4 hrs. or less, and contains the card on the 64-hole includes specific culture media, containing substrate special for biochemical tests, also includes one hole which contains culture media as negative control, this card completes a number of conventional tests, which have been modified be used within the VITEK-2 Compact systems .

2.5. Determination of minimal inhibitory concentration of antibiotics using the vitek- 2 compact system.

Use a system compact Vitek2 to determine the MICs For antibiotics directed at *Burkholderia* sp. isolates through the special label for the examination of the AST Card (Antibiotic sensitivity test card), According to the BioMerieux company's instructions, the antibiotic susceptibility test card contains 18 to 20 antibiotics distributed in 64 pits, each antibiotic having numerous concentrations, and the system reports changes in turbidity after the bacterium has grown .

2.6. Molecular Assays

2.6.1. Identification by PCR technique

1. Preparing the Primers Suspension

2. Polymerase Chain Reaction Protocol:-The DNA extract of *Burkholderia* sp. Isolates were subjected to antibiotic resistance genes by using PCR. The protocol was used depending on Promega Biosystems manufacturer's instruction. Single reaction (final reaction volume twenty µl) . All PCR components were assembled in PCR tube and mixed by refrigerated micro centrifuge at fifty *rcf* for ten second. Table(2).

Table (2) protocol of monplex PCR reaction mixture volumes

Mixture solution	
Master mix	8μL
DNA template	5μL
Forward primers	1.5μL
Reverse primers	1.5μL
Deion died water (d d water)	4μ L
	Final volume 20 μL

3. Extraction of bacterial Genomic DNA by boiling.

Total DNA was extracted from culture broth, 600 μl of culture broth pipetted into Eppendorf tubes then centrifuged at 4,300 x g for five min and the supernatant discarded; 200 μl of TE buffer was added, vortexed well, boiled for ten min by water bath on 100 degree, and then put on ice immediately for 1 min; this was centrifuged again at 6,700 xg for ten min and the supernatant was collected, which contains DNA for use as DNA template

4- Estimation of DNA Concentration

Nano Drop instrument was used to estimate DNA concentration and purity, one μl of each DNA sample was applied in Nano drop to measuring the optical density (O.D) at wave length 260nm and 280nm. An O.D of one corresponds to approximately 50μg/ml for double strand DNA. The final concentration of DNA was calculated according the formula:

$$\text{DND concentration } \mu\text{g/ml} = \text{O.D}_{260\text{ nm}} \times 50\mu\text{g/ml} \times \text{Dilution factor}$$

Also the purity calculated according to the formula:

$$\text{DNA Purity} = \text{O.D.}_{260\text{nm}} / \text{O.D.}_{280\text{nm}} (\text{range}=1.8-2)$$

All these equation calculated digitally by Nano drop setup.

5. PCR Thermocycling Conditions

The PCR tubes were placed on the PCR machine and the right PCR cycling program parameters conditions were installed as in table (3).

Table (3) PCR program that apply in the thermocycler

Gene Name	Temperature (°C) / Time					Cycles Number
	Initial Denaturation / time	Denaturation	Annealing	Extension	Final Extension / time	
<i>rec A</i>	96 °C/4 min	96 °C for 1min	59 °C/1 min	72 °C/ 1min.	72°C/2min.	35
<i>16 s rRNA</i>	94 °C /5 min	94 °C/1min.	60 °C/1min.	72 °C /1 min	72°C /10min	30
<i>TEM</i>	95 °C/10 min.	95 °C /30sec	55.5°C/1min	72 °C /1 min	72°C /7min	30
<i>PRND</i>	95 °C /10 min.	95 °C/1 min	68 °C /1 min	72 °C /1 min	72°C/10min	30
<i>PLTC</i>	95 °C /2 min.	92 °C /2 min	67 °C/1 min	72 °C /1 min	74 °C/1min	30
<i>CciI</i>	94°C/5min	94 °C /30 sec	50°C /30 sec	72 °C/1min	72°C/7min	30
<i>OPcI</i>	94°C/5min	94 °C /30 sec	56°C /30 sec	72 °C/1min	72 °C/1min	30

6.PCR identification.**7.Amplification reaction mixture.**

8.Primers selection:-All primers in this study were synthesized by Bio Mérieux Company , France as following in Table(4).

Table(4): Primers used in this study.

Primer Type	Primer sequence (5'-3')	Amplicon size (bp)	Reference
<i>recA</i>	F 5ATGACCAATCCGACCGATCTCAA 3' R 5' TCAGTGCTTGCGITNIGGGCAGTT3'	429	(7)
<i>16S-rRNA</i>	F GACTCCTACGGGAGGCAGCAG R CTGATCCGCGATTACTAGCGATTC	1020	(15)
<i>PRND</i>	F 5'GGG GCG GGC CGT GGT GATGGA3' R 5'YCC CGC SGC CTG YCT GGT CTG 3'	786	(22)
<i>PLTC1</i>	F 5'AACAGATCGCCCCGGTACAGAACG 3' R5'AGGCCCGGACACTCAAGAACTCG 3'	438	(22)
<i>opcI</i>	5' GCC TTT GTC GGA CTG AGC3'F 5' GTC TGT GCG TTT CGA GAG3' R	612	(4)
<i>TEM</i>	5'CAGCGGTAAGATCCTTGAGA3' 5' ACTCCCCGTCGTGTAGATAA3'	643	(27)
<i>ccil</i>	5' TTG ATC GCG GTC GTA CGA 3' 5' ACC GCA ACG CTG GTA AAG3'	319	(4)

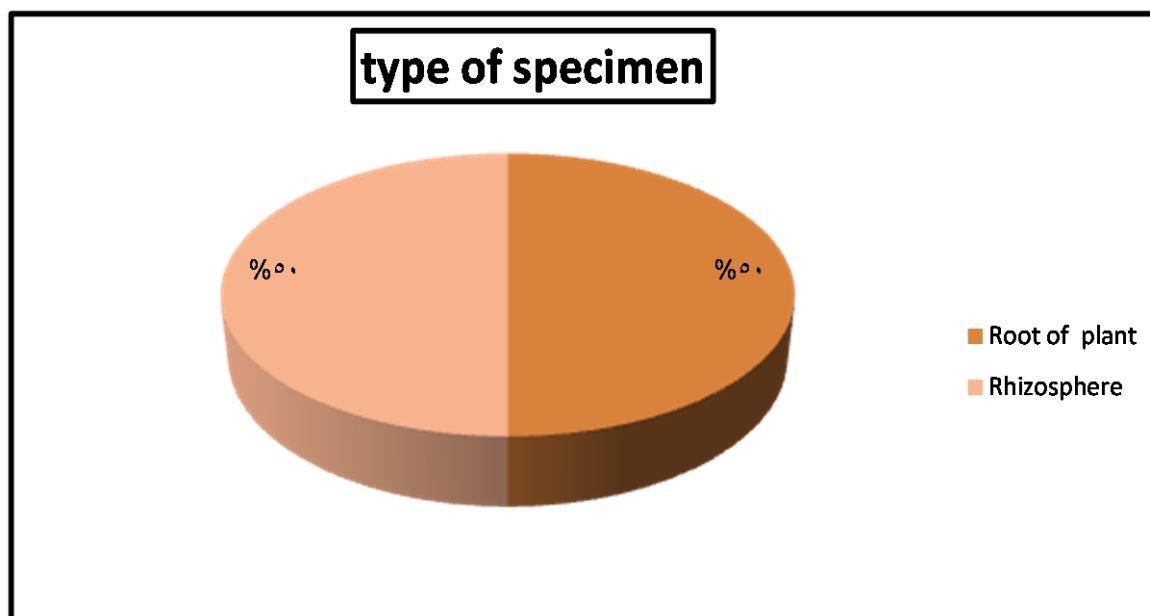
9. Detection of DNA content by agarose gel electrophoresis**A. Preparation of Agarose Gel and DNA Loading.****B.Sample preparation.****C.Agarose Gel Electrophoresis.****D.DNA sequencing of PCR products.****3. Results and Discussion****3.1.Specimens Collection**

In this study we were collecting of (90) environmental specimen include soil ,and the soil surrounding the root plant(Rhizospheres) in AL-Najaf province.All the samples were collected During a four-month period, from 1st October 2020 to January 2021. The isolates were obtained from in soil environment isolates were S₄1(2.2)%and S₅1(2.2)%observed in Figure (1).

3.2.Distribution and existences Burkholderia sp. in environment

In current study two isolates were obtained from Burkholderia sp. ,for the first time in Iraq, and the Middle East, the isolates of bacteria two new species(Mohammed(1) at the ratio 1(2.8)%and Mohammed(2) at the ratio 1(2.8)%have been recorder two from *Burkholderia* specie of in Gen Bank. from Were discovered near the roots of onion's (Rhizosphere plants and the surrounding soil ,not from fields, rice farms, palm trees, or ornamental plants, and were detected after study in home gardens. The effect of chemical fertilizers may kill bacteria, unlike the organic fertilizers that are used in home gardens in most cases, (21) mentioned The proportion of nutrient-solubilizing bacteria (nitrate, phosphate, potassium, iron, and zinc) isolated fromrhizosphere decreased with

inorganic fertilizer addition. and in other identical study for (5) isolated burkholderia sp. from palm rhizosphere, bacteria that solubilize phosphate (PSB) are helpful to plant growth. They live in a variety of soil habitats, including the root environment, there were existence of similarity between the



burkholderia strain which infect humans with isolate was present in the environment. this is explains why humans get infect despite good prevention efforts where transmission the bacteria from the soil to agricultural workers .Figure(1).

Figure (2): Distribution of The Environmental Specimens for genus *Burkholderia*

3.3. Identification of *Burkholderia* species

Initially identification of bacterial specimens which depended on some standards which involved Gram stain ,cultural ,morphology and biochemical tests .and Finally identification was performed with the automated vitek-2 compact system using GN-ID cards which contained 64 biochemical tests and one negative control .Exactly(90)isolates were performed identification and confirmed via vitek-2 compact system by usingGN-ID cards.All specimens were cultured on MacConkey agar and blood agar then suspected isolates were cultured on(OFPBL) which is the recommended medium for primary isolation of *Burkholderia sp.*All isolates from (OFPBL) agar medium confirmed using conventional diagnostic methods, then using GNcard and Vitek 2 system.All the suspected isolates obtained were microscopic examination under oil immersion shown Ge-ve rod bacteria under the microscope after staining with gram stain, they appeared as Ge-ve single short bacilli ,motile and they have been found to possess a polar flagella as well as pili used for attachment. ,On MacConkey agar medium, *BCC* isolates appeared as (NLF), small pale pink colonies, after four-seven days, colonies became dark pink to red due to oxidation of lactose ,this result agree with .⁽²⁵⁾ Figure (2).

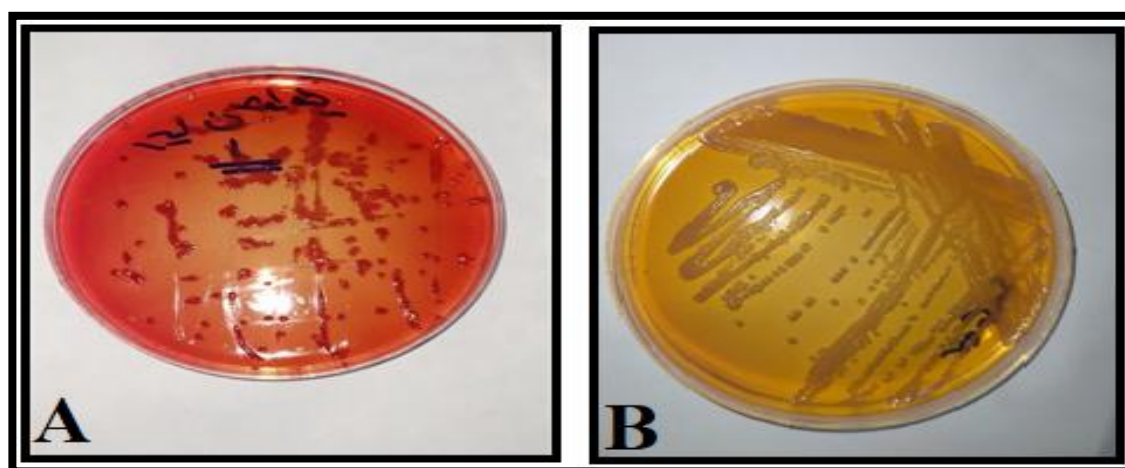


Figure (2): (A) *B.cepacia* colonies on MacConkey agar after 24 hour (B) *B.cepacia* colonies after 4-7 days.

B. cepacia isolates appeared on blood agar as smooth, slightly raised, and were alpha -hemolytic colonies. Figure(3) use the medium OFPBL for isolation primary was for *B.cepacia* the appropriate medium for its growth where the *B.cepacia* isolates appeared as smooth, yellow colonies that alter the medium color to yellow which refers to lactose utilization in medium . Among the OFPBL agar components is the casein enzyme hydrolysate which provides the necessary nitrogenous compounds and contains lactose which serves as carbohydrate source. Lactose sugar is readily utilized by *B.cepacia* complex The fermentation of lactose results in the release of acid end-products which Changes the color of medium to yellow and providing the Bcc colonies their yellow coloration. The selectivity of medium is due to the presence of antibiotics; bacitracin and polymyxin B , which together inhibition growth the bacteria flora present in respiratory secretions and sputum and for the inhibition of gram-positive organism⁽¹¹⁾.

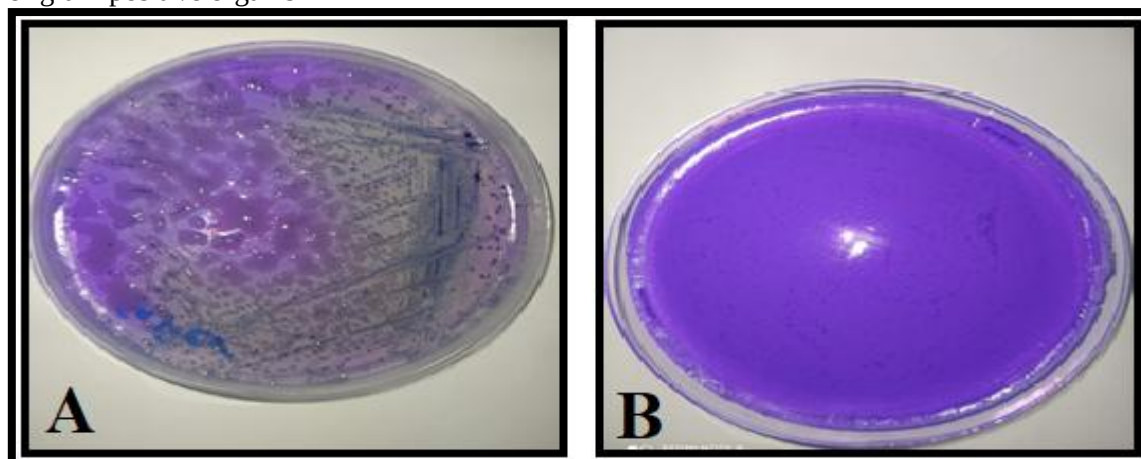


Figure (3): (A) shows isolation growth *Burkholderia* sp. on OFPBL agar after 24 hour.(B)Control treatment).

3.4.Diagnostic tests and general characteristics

The table (5) show the biochemical testing for isolation, the results were conform to general features, all isolates were positive (+) for oxidase and catalase test excepted the *B.cepacia* that isolate from the soil environment. this results obtained in current studying but negative result for production urease , VogesProskauer, methyl red test and H₂S production test. All the result were similar with⁽¹⁴⁾. isolates were able to ferment sugar on kligler iron agar, it produced (Alkaline or Acidic on slant and bottom (acidic) with H₂S formed.

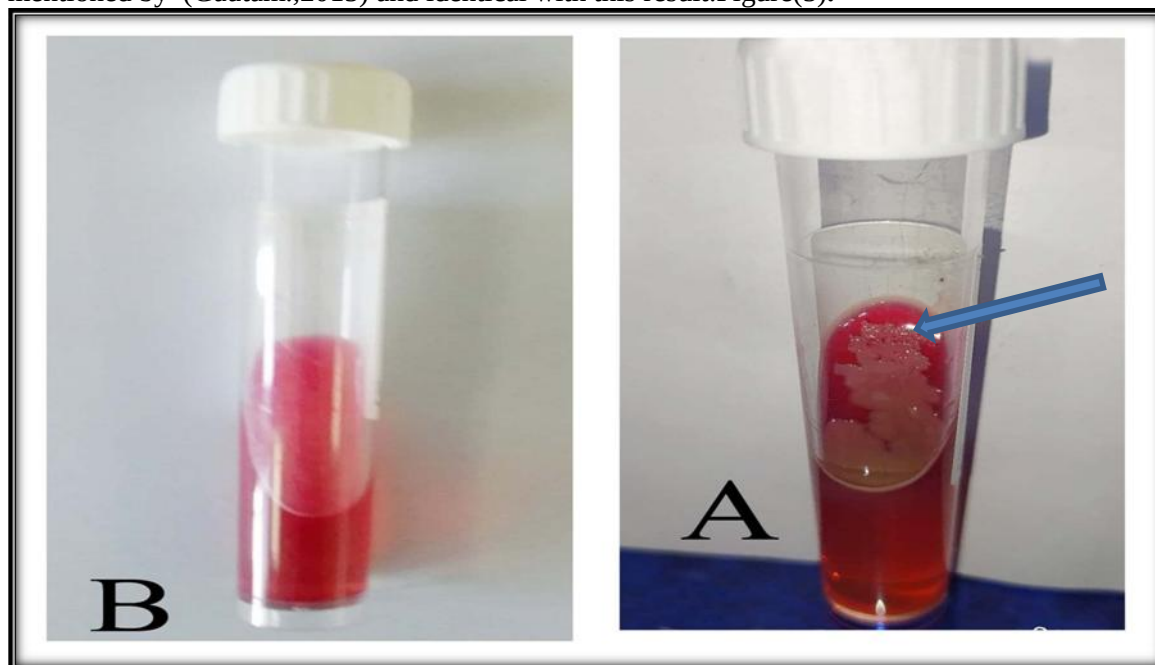
Alike study showed by⁽²⁾who had mentioned that some of *Burkholderia* sp. isolates are weakly oxidase positive and this is in similar with the results of our study. The enzyme catalase, is produced by bacteria during that breathe using oxygen, this enzyme is responsible for the breaking down of H₂O₂ into water and oxygen, so it is neutralizes the bactericidal effects of H₂O₂ and safeguard the bacterial cells. All of *Burkholderia* sp. isolates under study appeared indole test negative (-) and this result is in agreement with⁽³⁾who mentioned that bacteria hastryptophan's enzyme. the growth of *B. cepacia* isolates from environmental on TSI medium was K/A which indicates oxidation of glucose and produce acid from it. but produce H₂S in medium.

Table (5): Conventional biochemical tests suspected isolates.

Number isolate	Biochemical test							
	oxidase	catalase	motility	citrate	TSI	Odor smell	indol	urease
S ₄ (environmental)	-	-	+	+	K/A/-/+	dirty	-	-
S ₅ (environmental)	-	-	+	+	K/A/-/+	dirty	-	-

Positive : (+) Negative : (-)

some types *Burkholderia* sp. produced for Bright yellow pigment is on triple sugar iron agar (TSI) mentioned by (Gautam.,2015) and identical with this result. Figure(5).



Figure(5): (A) pigment production by isolate number 1 on TSI medium after 2 days.(B) TSI medium without growth(control treatment).

Burkholderia sp. different in ability to grow on culture medium, where *B. cepacia* were able for to growing in cetrimide medium and tolerate the high concentration of cetrimide in this medium. Some *Burkholderia* sp. Can produced a number of non-fluorescent pigments, including yellow pigments and

greenish-yellow diffusible pigments ⁽²⁰⁾.this study agree with themFigure(6)..

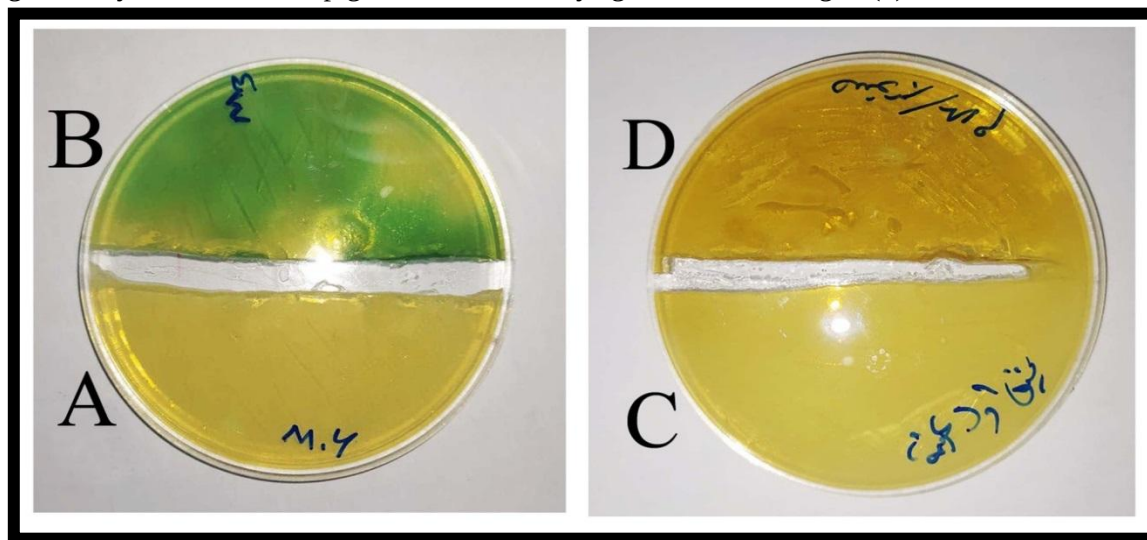


Figure (6): (D) Isolation of *burkholderia* sp from soil growth on nutrient agar with yellow diffusible pigment ,(C) Non detergent bacteria growth on same agar (no change in color) (B) isolation *burkholderia* sp from clinical growth on nutrient agar with green diffusible pigment, (D) Non detergent bacteria growth on same agar (no change in color).

3.4.Results obtained by ID GNB cards and VITEK 2 System

All of the suspected isolates were identified by Vitek 2 system, .All of the suspected isolates were identified by Vitek 2 system, the results showed that two *Burkholderia* spp. isolate were obtained in this study. showed the results of identification of the isolates by VITEK 2 system and ID GNB cards. For VITEK 2, confidence value is expressed by different categories of results: excellent (exc) (97%), very good (vg), good (g) (93-91%) and acceptable identification (88) % ,according to the manufacturing company.and only two isolate showed unidentified organism acceptable identification value. All the *Burkholderia* spp isolates 100% showed positive result for D-GLUCOSE (dGLU)test.

3.5. Molecular identification of *Burkholderia* sp

3.5.1.Genomic DNA Extraction:-Genomic DNA was successfully extracted from *burkholderia* sp. isolates by boiling method. Theconcentration and purity of extracted DNA were directly determined by spectrophotometry, extracted DNA purity ranged between (1.7-2). Extracted DNA were confirmed and analyzed by gel electrophoresis.

3.5.2.Identification of *burkholderia* sp. by *rec A* gene and polymerase chain reaction technique.

PCR assay resulted highly specific for each of the *Burkholderia* species tested, The gene *rec A* was detected successfully by using PCR technique with used specific primer and dye, and the result showed that all *burkholderia* spp tested isolates harbor the *recA* gene and this result confirms the presumptive identification of isolates by VITEK 2 System and ID GNB Card, performed during this study. As a matter of fact, *recA* gene encoding an enzyme involved in homologous recombination could be widely applied in bacterial systematics and has proven to be very useful for the identification of bacterial isolates ⁽⁴⁾.

The *rec A* gene has demonstrated to be an effective, objective, and portable taxonomic tool which can be used for both identification and classification. They found that the *rec A* genes of these bacteria were traditionally 98–99 percent similar within *B. cepacia* complex species and 94–95 percent similar between different *B. cepacia* complex species in several studies. ⁽²⁸⁾

3.5.3. Identification of *B.cepacia* selective isolates by universal 16A r RNA gene sequencing.

Burkholderia sp. selected isolates understudy were submitted to detect 16S rRNA gene with specific forward and reverse primers (356 F and 1020 R) by PCR technique and agarose gel electrophoresis, the length of amplified fragments were 1020 bp, DNA sequencing of the amplified fragments of PCR product which were equivalent to expected amplification size and nucleotide DNA sequence in each of the all isolates that showed positive result by PCR technique were performed at (Macrogen Company, Seoul, South Korea). Data of sequenced five isolates were analyzed and edit and the edited sequence were compare and alignment with data base using BLAST (Basic Local Alignment Search Instrument) from the (NCBI) website (<http://www.ncbi.nih.gov/Blast>), to find the closed relationship with submitted sequences. The use of 16S rRNA gene sequencing to study the relatedness of prokaryotic species is well established and has led to increased availability of 16S rRNA databases. The convergence of these technical and computational advances has also enhanced the application of 16S rRNA gene sequence analysis to bacterial identification. Many authors used 16S rRNA gene sequencing for identification *B.cepacia* environmental isolates. Another study done by ⁽²³⁾ they use 16S rRNA gene sequencing for identification several clinical pathogenic bacterial species included *Bcc* clinical isolates. In summary, we consider that this method provides accurate identification of *Bcc* genomovars from culture. In this study, after sequencing analysis, 2 isolates of *burkholderia* sp. have been submitted in Gen Bank under the accession number (MW632928) and (MW641983).

3.6. Molecular identification of virulence factors of *burkholderia* sp.

All gene above can be seen in Table (6) : Figure(7).

Table (6) Describe Of Genes For Isolates under Study.

Number isolate	Genes in this study						
	16s rDNA	rec A	PrnD	pltC	opcl	blaTEM	cciI
S ₄ (environmental)	+	+	+	+	+	-	-
S ₅ (environmental)	+	+	+	+	+	-	-

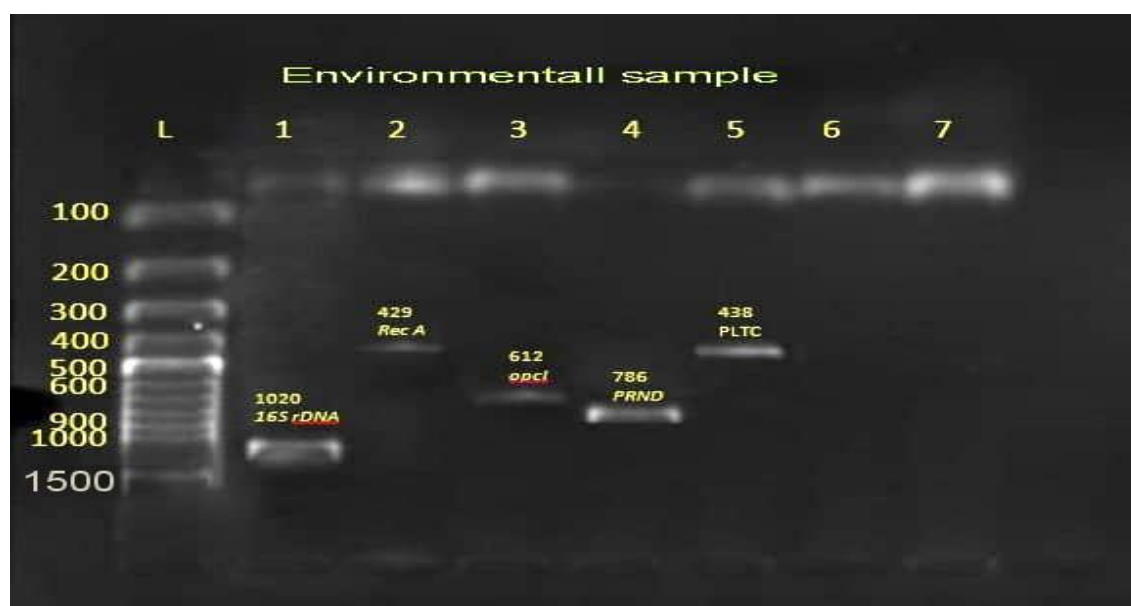


Figure : (7) Agarose gel electrophoresis (1% agarose, 75 V, for 1.5 hour) of seven genes (Diagnosis and virulence factors) of *Burkholderia* sp. from environment, stained with ethidium bromide and visualized on

a UV trans illuminator. Lanes (1, 2, 3, 4, 5,6,7 showed the seven genes in *Burkholderia sp.* isolates), lane L showed (100-1500bp) size DNA ladder.

3.7. Studying internal growth factors

3.7.1. The effects of various temperatures and pH on growth *burkholdria sp.*

Environmental factors effect on microbial community growth such as salinity, temperature, pH, or radiation, always takes precedence. as temperature different and pH value of the environment are the major factors affecting growth rate of a clinical and environmental isolates of *Burkholderia sp.* and protein stability ⁽²⁴⁾. All the strains of *burkholderia sp.* were grown at degrees temperature ((30,35,42)C° and there was no growth at temperature (46)C°, also observed that the bacteria had grown in degrees, PH (9), clearly and less at PH (5;6) and the absence growth at the PH(10) degree, this results agree with ⁽¹⁹⁾. Table(7).

Table(7) Show Influence of various temperature and pH on growth for *Burkholderia sp.*

Number Isolates	Growth on 30 C°	Growth on 35 C°	Growth on 42 C°	Growth on 46 C°	pH5	pH6	pH9	pH10
					Acidic	Acidic	Alkaline	Alkaline
S ₄	Growth	Growth	Growth	No	No	Weak	Growth	No
S ₅	Growth	Growth	Growth	No	No	Weak	Growth	No

3.8. Antibiotic Susceptibility Of *Burkholderia sp.* Isolates

3.8.1. Results of disk diffusion Test (DDT)

the samples taken from the soil showed sensitivity to most antibiotics, and the samples taken clinically were of high virulence. This indicates that these bacteria acquired the resistance genes. Or activation of resistance genes for antibiotics and there study conducted by ⁽²⁶⁾ who had found out that their *burkholdria sp.* isolates were resistant to Azithromycin and this result was in agreement with the results of this study when all isolates of *Burkholdria sp.* isolates under study showed resistance for this antibiotic. The result of mutation in its own DNA or Horizontal Gene Transfer acquisition of resistance-conferring DNA from another source where genetic material contained in small packets of DNA can be transferred between individual bacteria of the same species or even between different species. Table (8)

Table (8) Antibiotics resistance of *Burkholderia sp.* isolates.

Antibiotic	AMC	CTX	CAZ	P 10	IPM	AK	AZM	TOB	CRO	GN
Name of Isolate										
S ₄ Environmental	R	S	S	S	S	R	R	S	S	S
S ₅ Environmental	R	R	R	R	S	R	R	S	R	S

AMC=Augmentin ,AK=Amikacin CTX=Cefotaxime , AZM=Azithromycin,
CAZ= Ceftazidime ,TOB= Tobramycin ,P10= Penicillin ,CRO=Ceftriaxone

IPM=Imipenem , GN=Gentamicin

3.9. Determination of minimal inhibitory concentration(MIC) of antibiotics using the vitek 2 compact system. Use the system Compact 2 Vitek device to determine the minimum inhibition concentrations (MICs) of 10 antibiotics, with the AST Test Card and as directed by the manufacturer BioMeriex .The results showed that the MIC value for imipenem $16 \leq \mu\text{g} / \text{ml}$ with ratio 55.5%, and antibiotic Meropenem $16 \leq \mu\text{g} / \text{ml}$ with ratio 44.4 %, antibiotic Tazobactam $\geq 128 \mu\text{g} / \text{ml}$ with ratio 55.5 %.⁽⁹⁾ mention 71 per cent resistant to meropenem and 59 per cent to ceftazidime, Khurana, (2020) mention 21 % of *Burkholderia* sp.resistant for colistin, Thibault, F. M 2004 mention 50% of *Burkholderia* resistant to Ciprofloxacin and 16% resistant for Aztreonam,⁽¹⁾ showed *B.cepacia* isolates high resistance to gentamicin with 88 %.**Table:(9).**

Table:(9): Percentages of the value of the minimum inhibitory concentration antibiotic MIC

Resistant			Intermediate		Sensitive	
Antibiotic Name	Name.isolates	Percentage%	Name.isolates	Percentage%	Name. Isolates	Percentage%
clavulanic acid	4	44.4	0	0	5	55.5
Tazobactam	5	55.5	1	11.1	3	33.3
Ceftazidime	4	44.4	1	11.1	4	44.4
Cefepime	5	55.5	0	0	4	44.4
Aztreonam	5	55.5	2	22.2	2	22.2
Imipenem	5	55.5	1	11.1	3	33.3
Meropenem	4	44.4	0	0	5	55.5
Tobramycin	5	55.5	0	0	4	44.4
Ciprofloxacin	5	55.5	0	0	4	44.4
Cefazolin	4	44.4	0	0	5	55.5
Gentamicin	4	88	0	0	0	0

3.10.Detection of some virulence factors for the *Burkholderia* sp. Isolates

Table(16)shows the capability of nine *Burkholderia* Sp. isolates to produce different virulence factors. And The capability of isolated bacteria to cause infection as a result of their virulence factors, which play a direct and indirect role in infection and host body damage.

Table(10): Results of Studying Some Virulence Factors for *Burkholderia* sp.Isolates.

Name of Isolate	S ₄	S ₅
Virulence factor		
Urease	-	-
Biofilm	+	+
Gelatinase	+	+

Positive = (+) Negative = (-)

3.10.1.Urease and Gelatinase enzymes production

According to the results of the study, for two type *Burkholderia* sp.were unable to produce Urease enzyme. This results was in agreement with the results of current study. and non-identical with ⁽⁸⁾While produce Gelatinase enzymes Table(10).

3.10.2Biofilm formation:-Results obtained by CRA method in this study showed that two of *Burkholderia* spp isolates had the ability to produce biofilm from .Biofilm formation is one of the most common strategies for bacterial survival in harsh environments.Tube adherence, CRA,and Tissue culture plate were used to detect the production of biofilm by *Burkholderia* sp.(⁽¹⁷⁾).Table(10).

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